MOLECULAR AND CELLULAR BIOLOGY, Sept. 1990, p. 4623–4629
0270-7306/90/094623-07$02.00/0

Vol. 10, No. 9

Phosphorylated Forms of GAL4 Are Correlated with Ability To Activate Transcription

LAWRENCE M. MYLIN,[1] MARK JOHNSTON,[2] AND JAMES E. HOPPER[1,3,4]*

Department of Biological Chemistry,[1] Cell and Molecular Biology Program,[3] and Graduate Program in Genetics,[4] The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033, and Department of Genetics, Washington University, St. Louis, Missouri 63110[2]

Received 7 March 1990/Accepted 15 June 1990

$GAL4_I$, $GAL4_{II}$, and $GAL4_{III}$ are three forms of the yeast transcriptional activator protein that are readily distinguished on the basis of electrophoretic mobility during sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Phosphorylation accounts for the reduced mobility of the slowest-migrating form, $GAL4_{III}$, which is found to be closely associated with high-level *GAL/MEL* gene expression (L. Mylin, P. Bhat, and J. Hopper, Genes Dev. 3:1157–1165, 1989). Here we show that $GAL4_{II}$, like $GAL4_{III}$, can be converted to $GAL4_I$ by phosphatase treatment, suggesting that in vivo $GAL4_{II}$ is derived from $GAL4_I$ by phosphorylation. We found that cells which overproduced GAL4 under conditions in which it drove moderate to low levels of *GAL/MEL* gene expression showed only forms $GAL4_I$ and $GAL4_{II}$. To distinguish which forms of GAL4 ($GAL4_I$, $GAL4_{II}$, or both) might be responsible for transcription activation in the absence of $GAL4_{III}$, we performed immunoblot analysis on UASgal-binding-competent GAL4 proteins from four *gal4* missense mutants selected for their inability to activate transcription (M. Johnston and J. Dover, Proc. Natl. Acad. Sci. USA 84:2401–2405, 1987; Genetics 120;63–74, 1988). The three mutants with no detectable *GAL1* expression did not appear to form $GAL4_{II}$ or $GAL4_{III}$, but revertants in which GAL4-dependent transcription was restored did display $GAL4_{II}$- or $GAL4_{III}$-like electrophoretic species. Detection of $GAL4_{II}$ in a UASgal-binding mutant suggests that neither UASgal binding nor *GAL/MEL* gene activation is required for the formation of $GAL4_{II}$. Overall, our results imply that $GAL4_I$ may be inactive in transcriptional activation, whereas $GAL4_{II}$ appears to be active. In light of this work, we hypothesize that phosphorylation of $GAL4_I$ makes it competent to activate transcription.

How eucaryotic transcriptional activators work is an unsolved problem. One eucaryotic transcriptional activator that has been subject to considerable study is the GAL4 protein, which activates transcription of the galactose/melibiose regulon genes (*GAL/MEL* genes) in the yeast *Saccharomyces cerevisiae* (for a review, see reference 19). In cells grown in the absence of galactose and glucose, GAL4 binds specifically to sites (UASgal) located upstream of the *GAL/MEL* gene promoters (2, 10, 11, 14, 27, 42, 48) but cannot activate transcription as a result of its association with the GAL80 protein (23, 28, 30, 37, 45, 49). Presentation of galactose (in the absence of glucose) relieves *GAL80* inhibition, allowing *GAL4* to activate *GAL/MEL* gene transcription. Glucose represses GAL4 protein-dependent transcription (even in the absence of the GAL80 protein [45, 49; reviewed in reference 13]) by mechanisms which include reduced GAL4-UASgal interaction (14, 27, 42).

Learning how GAL4 activity is modulated by carbon source has become a central aim in the overall goal of understanding how it activates transcription. Recently, we discovered that GAL4 protein exists in multiple forms that can be distinguished on the basis of electrophoretic mobility during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (33). The slowest-migrating form, $GAL4_{III}$, is phosphorylated and is found to be closely associated with increased *GAL/MEL* gene expression (33). Two other GAL4 species, $GAL4_I$ and $GAL4_{II}$, are detected in extracts of noninduced cells. Within 30 min after galactose addition, $GAL4_{III}$ appears at the expense of $GAL4_{II}$ and $GAL4_I$. $GAL4_{III}$ is not detected, however, after long-term growth on galactose-containing medium, a condition characterized by lower than maximal *GAL/MEL* gene expression (45) and the presence of $GAL4_{II}$ and $GAL4_I$ (33). Addition of glucose to newly induced cells that contain $GAL4_{III}$ triggers rapid removal of GAL_{III} and the reappearance of $GAL4_{II}$ (33). These results, and the fact that $GAL4_{III}$ is converted to $GAL4_I$ by phosphatase treatment in vitro, led us to propose that phosphorylation state changes of GAL4 are key to modulating its activity (33).

The overall results from the previous work suggested that phosphorylation of GAL4 to $GAL4_{III}$ might make it a better transcriptional activator. In particular, the fact that only $GAL4_I$ and $GAL4_{II}$ were detected in long-term galactose-grown cells suggested that $GAL4_{III}$ might not be required for *GAL/MEL* gene transcription and that one or the other (or both) of these forms is active. To test this notion and to investigate further the physical and functional significance of the GAL4 isomers, we have initiated a genetic and physical analysis of $GAL4_I$ and $GAL4_{II}$. Here we show that $GAL4_{II}$, like $GAL4_{III}$, can be converted to $GAL4_I$ in vitro by phosphatase treatment, suggesting that $GAL4_{II}$ is derived from $GAL4_I$ by phosphorylation. We also report that overproduction of $GAL4_I$ and $GAL4_{II}$ under conditions yielding no detectable $GAL4_{III}$ brings about *GAL/MEL* gene expression. We show that three *gal4* missense mutants blocked in the GAL4 transcription activation function, but not specific DNA binding, do not produce detectable levels of $GAL4_{II}$ and $GAL4_{III}$ and that GAL4 heterogeneity is restored in revertants with restored activity. However, a mutant GAL4 protein that does not bind to UASgal exhibits $GAL4_{II}$ but not detectable amounts of $GAL4_{III}$.

* Corresponding author.

MATERIALS AND METHODS

Yeast strains, plasmids, and growth media. Yeast strains Sc252 (SJ21R [22]) and Sc285 (*gal80*-deletion derivative of Sc252 [33]) have been described previously. Yeast strains Sc413 and Sc414 are derivatives of strain Sc252 that bear chromosomal deletions of the *GAL4* or *GAL80* gene or both and are described elsewhere (33, 34). Yeast strain Sc340 expresses elevated levels of GAL4 protein upon galactose addition from a *GAL10p-GAL4* fusion gene integrated at the *his3* locus (34, 41). Strains Sc490 and Sc491 were created by transformation of strains Sc252 and Sc285, respectively, with *Bam*HI-digested pJJ118 (3). pJJ118 contains a *his3*::*ADH1-GAL4-URA3*::*his3* cassette than can be excised by *Bam*HI digestion and integrates at the *HIS3* locus by homologous recombination (3, 40). The *ADH1* (*ADC1*) promoter is described elsewhere (1). Ura^+ transformants were screened by Western blotting (immunoblotting) to confirm increased production of GAL4 protein. Strain Sc541 was produced from Sc385 (*gal3* deletion in Sc252 [3a]) by the same procedure.

Variants of yeast strain Sc252 bearing chromosomal disruptions of the *TRP1*, *GAL4*, and *GAL80* genes were produced by one-step plasmid-directed gene replacement (18, 40), using plasmid pOH15R-T-UD (a gift of Dahlkyun Oh). Plasmid pOH15R-T-UD is a pBR322-based vector (5) that carries a derivative of the *TRP1*-containing 1.4-kilobase-pair *Eco*RI fragment from YRP7 (47). The *TRP1* gene in pOH15R-T-UD is nonfunctional because of insertion of the 1.1-kilobase-pair *Hin*dIII *URA3*-containing fragment from YEp24 (7). Yeast strains Sc545 and Sc546, respectively, were created by transformation of strains Sc413 (*gal4D GAL80*) and Sc414 (*gal4D gal80D*) with *Eco*RI-digested pOH15-T-UD. Ura^+ transformants were recovered and screened for the Trp^- phenotype.

The *gal4* mutant and *GAL4* pseudorevertant alleles characterized here were described previously (20, 21). Plasmid names are used here in place of the original allele numbers. Descriptions are given in the text, figures, and tables.

Plasmid YCP50CBN (a gift of Wajeeh Bajwa) was constructed from YCP50 (25) by removal of sequences separating the *Bam*HI and *Nru*I and the *Eco*RI and *Cla*I sites of YCP50, with elimination of the *Nru*I and *Cla*I sites. Plasmids used for yeast transformations were amplified in *Escherichia coli* HB101 (8) and manipulated according to standard procedures (32).

The buffered 5× growth medium used for most experiments has been described elsewhere (33, 34). Unbuffered 1× synthetic growth medium was described previously (16). Preparation and use of the carbon sources glycerol and lactic acid were previously described (45). Dextrose and glucose-free galactose were obtained from Pfanstiel Laboratories (Waukegan, Ill.).

Yeast cell extract preparation and Western blotting of GAL4 protein. Yeast cells were disrupted by vortexing with glass beads, pelleted, and extracted in 1× electrophoresis sample buffer essentially as described previously (33). The rabbit serum used for Western blotting was previously characterized (33, 34). The GAL4 protein electrophoretic standard appears elsewhere (33).

Radiolabeling and immunoprecipitation of GAL4 protein from yeast cells. The conditions used for radiolabeling of yeast cells in culture with [^{35}S]methionine were essentially as described elsewhere (33), with the following modifications. Cells were pregrown (optical density at 600 nm [OD_{600}] of 0.2 to 0.3) in labeling medium initially supplemented with 0.5% (wt/vol) dextrose. Before labeling, the cells were resuspended at a higher density in fresh medium lacking dextrose. Cells were harvested by centrifugation at 5,000 rpm for 10 min at room temperature in a Sorvall HS4 rotor, resuspended to a final OD_{600} of 0.4 in prewarmed labeling medium, and incubated for another 30 min before galactose addition. Galactose was added to a final concentration of 2% (wt/vol). Isotope was added after 1 h of galactose induction, and labeling was allowed to proceed for 1 h. The chase period with 1 mM unlabeled methionine was restricted to 5 min. Cells were chilled and harvested, and extracts were prepared and immunoprecipitated as described previously (33).

Enzyme assays. All determinations were performed in triplicate. Galactokinase assays (4) were performed on homogenates of yeast cells prepared by disruption with glass beads (6) and clarified by centrifugation at 10,000 × *g* for 10 min at 4°C. Protein estimations were done by the method of Bradford (9), using reagent purchased from Bio-Rad Laboratories (Richmond, Calif.). α-Galactosidase assays were performed on unfractionated yeast cell homogenates (38). Protein assays for these reactions were performed according to Hess et al. (15).

RESULTS

$GAL4_{II}$ is a phosphorylated form of GAL4 protein. It was previously reported that the electrophoretic species $GAL4_{III}$ differs from $GAL4_I$ by phosphorylation: alkaline phosphatase treatment increases its electrophoretic mobility to that of $GAL4_I$ (33). The results from experiments using the protein synthesis inhibitor cycloheximide and Western blot analysis of GAL4 protein suggested a possible product-precurser relationship between $GAL4_I$ and $GAL4_{II}$ as well as $GAL4_{II}$ and $GAL4_{III}$ (33). We sought to determine more directly whether $GAL4_{II}$ differs from $GAL4_I$ by phosphorylation. We assayed for increased electrophoretic mobility of $GAL4_{II}$ after in vitro phosphatase treatment of immunoprecipitated in vivo ^{35}S-labeled GAL4 as described previously (33).

Yeast strain Sc541 was used as the source of in vivo ^{35}S-labeled GAL4 for these experiments. This strain substantially overproduces GAL4 protein from an *ADH1-GAL4* construct integrated in *HIS3* (3, 23, 33) but produces only forms $GAL4_I$ and $GAL4_{II}$ during short-term galactose growth (Mylin and Hopper, unpublished data) because of a defective *GAL3* gene which delays transcription induction (46). We used the *gal3* deletion to facilitate the isolation of radiolabeled $GAL4_{II}$ under the culture conditions used previously to isolate ^{35}S-labeled $GAL4_{III}$. Use of the *gal3* deletion here is valid in that *GAL3* function is not directly required for formation of $GAL4_I$, $GAL4_{II}$, or $GAL4_{III}$. That is, $GAL4_I$, $GAL4_{II}$, and $GAL4_{III}$ are detected in *gal3D gal80D* cells under the appropriate growth conditions (Mylin and Hopper, unpublished data).

Qualitatively different patterns of GAL4 protein were obtained when the *gal3*-deleted SC541 and nearly isogenic *GAL3* control strain SC490 were labeled in culture with [^{35}S]methionine in the presence of galactose (Fig. 1). The *gal3*-deleted strain produced only forms $GAL4_{II}$ and $GAL4_I$; the nearly isogenic *GAL3* control cells produced forms $GAL4_{III}$ and $GAL4_I$. Incubation of the GAL4 immunoprecipitates with calf intestinal alkaline phosphatase in the absence of added inhibitor increased the electrophoretic mobilities of both $GAL4_{II}$ and $GAL4_{III}$ to that of $GAL4_I$ (Fig. 1; compare lanes 5 and 6 and lane 7 and 8). We

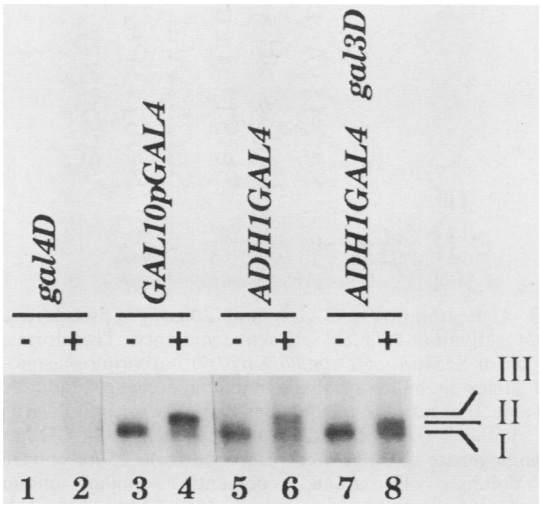

FIG. 1. Demonstration that incubation of $GAL4_{II}$ with alkaline phosphatase increases its electrophoretic mobility. Yeast strains Sc413 (*gal4D*; lanes 1 and 2), Sc340 (*GAL10p-GAL4*; lanes 3 and 4), Sc490 (*ADH1-GAL4*; lanes 5 and 6), and Sc541 (*ADH1-GAL4 gal3D*; lanes 7 and 8) were labeled in culture with [^{35}S]methionine (see Materials and Methods). Radiolabeled GAL4 protein was isolated by indirect immunoprecipitation from cell extracts and treated with calf intestinal alkaline phosphatase in the presence (+) or absence (−) of added inhibitor essentially as described previously (33). After electrophoresis, gels were prepared for fluorography as described previously (33), and exposed to Kodak XAR-5 film at −70°C for 22 h (lanes 3 and 4) or 14 days (all other lanes). Each lane on the autoradiogram represents GAL4 protein extracted from the equivalent of 2 ml of the labeling culture (OD_{600} = 0.4). I, II, and III indicate the positions of $GAL4_I$, $GAL4_{II}$, and $GAL4_{III}$, respectively.

conclude that $GAL4_{II}$ is a phosphoprotein and, like $GAL4_{III}$ (33), undergoes dephosphorylation to $GAL4_I$.

$GAL4_{III}$ is not required for low to moderate levels of transcription activation. $GAL4_{III}$ is not detected in wild-type cells grown long term on galactose (33). This raises the possibility that $GAL4_I$ or $GAL4_{II}$ provides the lower *GAL/MEL* gene activity characteristic of cells adapted to long-term growth on galactose. Alternatively, transcription activation in long-term galactose-grown cells could be due to levels of $GAL4_{III}$ below detection. To more rigorously test whether $GAL4_{III}$ is responsible for submaximal *GAL/MEL* gene expression, we sought to overproduce GAL4 protein under conditions in which it drives moderate levels of *GAL/MEL* gene expression in the absence of galactose and glucose. Such conditions are realized in uninduced (glycerol-lactic acid [Gly/Lac]-grown) *GAL80* cells in which the GAL4 protein is overproduced (23).

We overproduced the GAL4 protein by using an *ADH1-GAL4* construct (3, 23) integrated in the chromosome. The cells were grown in Gly/Lac medium and assayed for galactokinase and α-galactosidase activities, and their GAL4 forms were detected by an immunoblot. In *GAL80* cells bearing the *ADH1-GAL4* construct, the *GAL1* and *MEL1* genes were expressed at 4 and 74%, respectively, of the levels observed in the *GAL4 gal80* cells (Table 1). No $GAL4_{III}$ was evident in the *GAL80* cells despite substantial overproduction of $GAL4_I$ and $GAL4_{II}$ (Fig. 2, lane wt). Overproduction of GAL4 did not interfere with the formation of $GAL4_{III}$ since either the addition of galactose (data not shown) or the deletion of *GAL80* (Fig. 2, lane gal80D) allowed for abundant $GAL4_{III}$ formation. These results

TABLE 1. Galactose-independent *GAL1* and *MEL1* expression in cells expressing elevated levels of GAL4 protein

Genotype[a]	Expression			
	Without GAL4 overproduction[b]		With excess GAL4 protein[c]	
	GAL1	*MEL1*	*GAL1*	*MEL1*
GAL4 GAL80	<0.5	4	4	74
GAL4 gal80D	100[d]	100[d]	128	164
gal4D GAL80	<0.5	<0.5	ND[e]	ND
gal4D gal80D	<0.5	<0.5	ND	ND

[a] Strains listed were derived from SC252 (SJ21R) by disruption of the *GAL4* or *GAL80* gene (Sc252, Sc285, Sc413, and Sc414; see Materials and Methods). Each was transformed to uracil prototrophy with the vector or construct indicated.

[b] Strains were transformed with the URA3$^+$ centromere vector YCP50 CBN.

[c] Strains contained an *ADH1-GAL4* fusin gene integrated at the *his3* locus (see Materials and Methods).

[d] Transformants were grown in 5× synthetic Gly/Lac medium lacking uracil. Enzyme activities are expressed as percentages of the levels obtained in the *gal80*-deletion strain transformed with the vector YCP50CBN. Such values are typically 1.5- to 2-fold higher than the levels found in nearly isogenic *GAL80* cells grown on galactose-containing medium (45).

[e] ND, Assays were not performed.

support the notion that $GAL4_{III}$ is not required for low to moderate level *GAL/MEL* gene expression and imply that $GAL4_I$ or $GAL4_{II}$ (or both) activates transcription.

***gal4* missense mutants defective in transcription activation are also deficient in $GAL4_{II}$ (and $GAL4_{III}$) formation.** To learn which forms of GAL4 might be transcriptionally active, we analyzed GAL4 protein produced from four *gal4* missense mutant alleles selected for their inability to activate *GAL1* expression in vivo (BM797, BM810, BM813, and BM814 [20, 21]). The amino acid alterations of these mutants are presented in Table 2. These mutant GAL4 proteins produced in *E. coli* exhibit near-wild-type affinity in vitro for UASgal (20). The four mutant alleles (each carried on a CEN vector [21]) were assayed for their ability to activate *GAL1* expression in the absence of galactose in cells deleted for both *GAL4* and *GAL80* (Table 2). Of the four, only the BM813 mutant (Ser-322 → Phe) activated detectable levels of galactokinase expression.

Western immunoblot analysis revealed a striking difference between the mutant and wild-type GAL4 proteins (Fig.

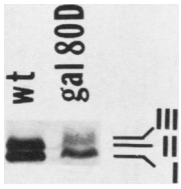

FIG. 2. Demonstration that partial relief of *GAL80*-mediated repression is not accompanied by detectable levels of $GAL4_{III}$. Yeast strains Sc490 (lane wt) and Sc491 (lane gal80D), each of which harbors an *ADH1-GAL4* fusion gene at the *his3* locus, were grown in 5× synthetic Gly/Lac medium lacking uracil. Cell homogenate pellet extracts were prepared, and volumes of extracts representing similar amounts of Coomassie blue-staining protein were analyzed by immunoblotting. Lane gal80D contains forms $GAL4_I$, $GAL4_{II}$, and $GAL4_{III}$, although the latter two were not well resolved in this assay. I, II, and III indicate the positions of electrophoretic forms $GAL4_I$, $GAL4_{II}$, and $GAL4_{III}$, respectively.

TABLE 2. *GAL1* expression in *gal4D gal80D* cells expressing plasmid-borne mutants of *GAL4*

Plasmid[a]	Type of GAL4 protein encoded[b]	Galactokinase activity[c]
BM292	Wild type	100
BM797	Ser-352 → Phe	<0.5
BM810	Ser-511 → Pro	<0.5
BM813	Ser-322 → Phe	5
BM814	Leu-331 → Pro	<0.5
BM1314	Ser-511 → Arg	98
BM1315	Leu-331 → His	67
BM1352	Ser-511 → Leu	95
BM1493	Ser-352 → Val	42
BM453	No GAL4	<0.5

[a] These plasmids carrying alleles described elsewhere (21) were introduced into *gal4D gal80D* yeast strain Sc546 (see Materials and Methods).

[b] Taken from Johnston and Dover (21); residues are numbered according to Laughon and Gesteland (26).

[c] Transformants were grown in synthetic Gly/Lac medium lacking tryptophan. Galactokinase activities were measured in cell extracts (see Materials and Methods) and are expressed as percentages of the levels measured in cells transformed with BM292.

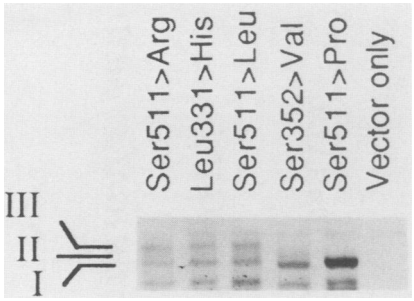

FIG. 4. Restoration of $GAL4_{II}$ and $GAL4_{III}$ in pseudorevertants of *gal4* activation-deficient missense mutants. Transformants of yeast strain Sc546 (*gal4D gal80D trp1D*) harboring plasmid-borne *GAL4* alleles encoding the single amino acid changes indicated (see also Table 2) or no *GAL4* sequences (lane Vector only) were grown in 5× synthetic Gly/Lac medium to a cell density of OD_{600} = 0.2. Cell homogenate extracts were prepared from 25-ml culture samples. Volumes of extracts representing similar amounts of Coomassie blue-staining protein were assayed by immunoblotting. I, II, and III represent the positions of $GAL4_I$, $GAL4_{II}$, and $GAL4_{III}$, respectively.

3). Only GAL4 protein species migrating with $GAL4_I$ were detected in extracts of *GAL80* cells bearing the three mutants that did not activate *GAL1* expression. For those three mutants, the lack of $GAL4_{II}$ correlated with the lack of GAL1 enzyme production. The mutant that produced low levels of galactokinase (BM813) displayed detectable but low amounts of $GAL4_{II}$. Addition of galactose did not alter the electrophoretic profiles of the mutant proteins expressed in *GAL80* yeast cells (Fig. 3). Similar electrophoretic profiles were obtained when the mutant *gal4* alleles were expressed in cells lacking the *GAL80* gene (data not shown).

These results reveal a striking correlation between $GAL4_{II}$ formation and transcriptional activation activity and imply that $GAL4_I$ is inactive in transcription activation.

GAL4 activity and heterogeneity ($GAL4_{II}$ and $GAL4_{III}$ formation) are reestablished in pseudorevertants. Johnston and Dover (21) isolated transactivation-competent revertants of three of the four activation-deficient *gal4* alleles. Several of these revertants are pseudorevertants since they contain non-wild-type amino acids at the original mutated sites (21). We transferred four such pseudorevertants into the SJ21R background and tested them for *GAL1* expression (Table 2) and GAL4 protein electrophoretic profile (Fig. 4). Two of the revertants (BM1352 and BM1314) expressed galactokinase at wild-type levels; the other two revertants (BM1315 and BM1493) express somewhat lower galactokinase levels. The three revertants with the highest levels of *GAL1* expression (Ser-511 → Arg, Ser-511 → Leu, and Leu-331 → His) all produced $GAL4_{III}$ (Fig. 4). In each case, the $GAL4_{III}$ species was replaced by $GAL4_{II}$ in glucose-grown *gal80*-deletion cells and in glycerol/lactic acid-grown *GAL80* cells (data not shown), as was previously demonstrated for the wild-type *GAL4* allele (33). By contrast, the Ser-352 → Val mutant did not exhibit detectable levels of $GAL4_{III}$ (Fig. 4). Instead, this mutant expressed a distinct $GAL4_I$ as well as GAL4 species that migrated as a diffuse

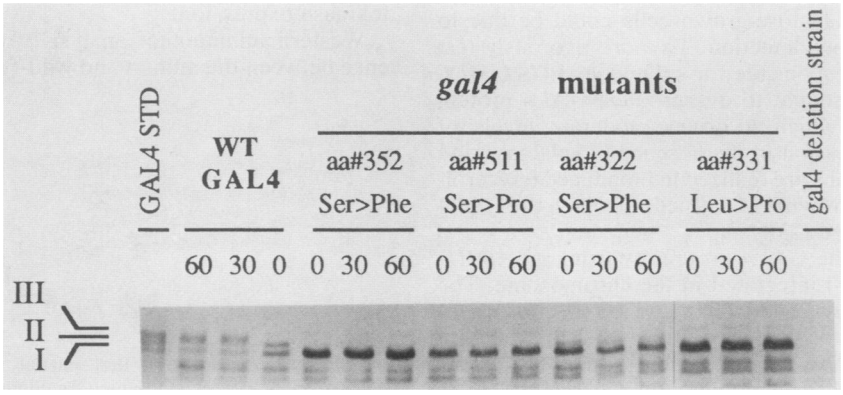

FIG. 3. Absence of $GAL4_{II}$ and $GAL4_{III}$ in *gal4* missense mutants defective in transcriptional activation. Transformants of yeast strain Sc545 (*gal4D GAL80 trp1D*) bearing plasmids encoding the wild-type *GAL4* allele or carrying *gal4* missense mutations were grown in 5× synthetic Gly/Lac medium lacking tryptophan to a cell density of OD_{600} = 0.2. Galactose was added to a final concentration of 2%, and incubation continued. Culture samples (25 ml) were taken immediately before galactose addition (lanes 0) and at 30 (lanes 30) and 60 (lanes 60) min after galactose addition. Cell homogenate pellet extracts were prepared, and volumes representing similar amounts of Coomassie blue-staining protein were assayed by immunoblotting. The predicted amino acid (aa) substitutions for the respective mutant proteins are indicated above the appropriate lanes (also see Table 2). Note that the order of the lanes representing the wild-type allele (WT GAL4) is reversed with respect that of the mutants. Extract from *gal4*-deletion cells lacking any plasmid is included as a control. I, II, and III indicate the positions of electrophoretic forms $GAL4_I$, $GAL4_{II}$, and $GAL4_{III}$, respectively in lane GAL4 STD.

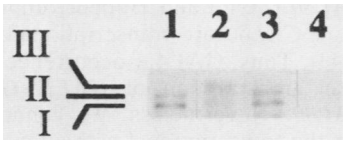

FIG. 5. Ability of a *gal4* mutant defective in specific DNA binding to $GAL4_{II}$. Yeast strain Sc545 (*gal4D GAL80 trp1D*) bearing plasmid BM292 (lane 1) and Sc546 (*gal4D gal80D trp1D*) bearing plasmids BM292 (lane 2), BM792 (lane 3), and BM453 (lane 4) were grown in 5× synthetic Gly/Lac medium lacking tryptophan to a cell density of OD_{600} = 0.2. See text for a description of BM792 (a DNA-binding mutant) and Table 2 for descriptions of the others (the wild-type allele or no *GAL4*). Cell homogenate pellet extracts were prepared from 25-ml culture samples. Volumes of extracts representing similar amounts of Coomassie blue-staining protein were assayed by immunoblotting. I, II, and III indicate the positions of electrophoretic forms $GAL4_I$, $GAL4_{II}$, and $GAL4_{III}$, respectively.

band with mobility intermediate to those of $GAL4_I$ and $GAL4_{III}$.

For all four pseudorevertants, the acquisition of transcriptional activity correlated with the reappearance of electrophoretic forms other than $GAL4_I$. Whether these forms represent phosphorylation states identical to the wild-type protein forms $GAL4_{III}$ and $GAL4_{II}$ remains unknown. In any case, these results are consistent with the idea that phosphorylated forms other than $GAL4_I$ activate low- to moderate-level *GAL/MEL* gene transcription in the absence of $GAL4_{III}$.

Neither UASgal DNA binding nor *GAL4* transcription activation is required for $GAL4_{II}$ formation. A *gal4* mutant altered in the DNA-binding domain (Glu for Lys at codon 17 [BM792]) was previously shown to have severely reduced affinity for UASgal in vitro (21). We used this mutant to determine whether binding to UASgal is required for $GAL4_{II}$ formation. The BM792 CEN vector was placed into a yeast strain harboring chromosomal deletions of both *gal4* and *gal80* genes. The altered GAL4 protein encoded by the BM792 allele does not activate *GAL1* expression in at least two genetically distinct backgrounds, strain YM2271 (congenic to S288C [21]) and the SJ21R background used here (less than 1% of wild type; data not shown). The altered GAL4 protein was present in yeast cells at levels comparable to that in a wild-type strain (Fig. 5). The electrophoretic pattern of GAL4 protein encoded by the BM792 mutant showed only forms $GAL4_{II}$ and $GAL4_I$ (Fig. 5, lane 3). By comparison, the wild-type GAL4 protein from Gly/Lac-grown *gal80* cells occurred typically as forms $GAL4_{III}$ and $GAL4_I$ (Fig. 5, lane 2; see also reference 33). We conclude from these results that phosphorylation to $GAL4_{II}$ does not depend on UASgal interaction and cannot be a result of *GAL* gene transcription.

DISCUSSION

We have presented the following results. First, treatment with alkaline phosphatase in vitro converts $GAL4_{II}$ to a form that comigrates during SDS-PAGE with $GAL4_I$. Second, *GAL80* cells which substantially overproduce GAL4 protein and thereby constitutively express the *GAL1* and *MEL1* genes at low to moderate levels exhibit only $GAL4_I$ and $GAL4_{II}$. Third, in *gal4* missense mutants with no detectable $GAL4_{III}$, *GAL1* expression correlates strictly with detection of GAL4 electrophoretic species other than $GAL4_I$. Finally, a *gal4* missense mutant that is defective in UASgal binding and does not activate *GAL/MEL* gene expression exhibits $GAL4_I$ and $GAL4_{II}$ but not $GAL4_{III}$.

The simplest interpretation of the in vitro phosphatase conversion of $GAL4_{II}$ to $GAL4_I$ is that in vivo, $GAL4_I$ gives rise to $GAL4_{II}$ by phosphorylation. Thus, both $GAL4_{II}$ and $GAL4_{III}$ (33) differ from $GAL4_I$ by phosphorylation. Previous Western blot analysis of GAL4 protein in yeast cell extracts revealed that the relative abundances of $GAL4_{II}$ and $GAL4_{III}$ were rapidly and inversely altered in cells undergoing galactose induction or glucose repression (33). Those results, together with the results presented here, imply that in vivo, $GAL4_{II}$ gives rise to $GAL4_{III}$ by phosphorylation and that $GAL4_{III}$ can be dephosphorylated to $GAL4_{II}$ (33). Although we have shown that $GAL4_{III}$ (33) and $GAL4_{II}$ (this work) can be converted to $GAL4_I$ in vitro by phosphatase treatment, we have not been able to achieve in vitro conversion of $GAL4_{III}$ to $GAL4_{II}$; in our assays, $GAL4_{III}$ is converted primarily to $GAL4_I$, with only a smear occurring throughout the $GAL4_{II}$ region (data not shown). We imagine that in vivo conversion of $GAL4_{III}$ to $GAL4_{II}$ is a closely controlled reaction that is not easily reproduced in vitro. Identification of the residues phophorylated in $GAL4_{II}$ and $GAL4_{III}$ will provide insight into the physical relationship between these species.

Previous work (33) showed that during long-term growth of *GAL80* cells on galactose-containing medium, GAL4 protein-dependent transcription does occur in the absence of detectable $GAL4_{III}$. Here we report another example wherein lower than immediate postinduction *GAL1* and *MEL1* gene expression occurs in the absence of detectable $GAL4_{III}$. We find that the low to moderate constitutive *GAL1* and *MEL1* expression characteristic of GAL4-overproducing, *GAL80* cells grown in Gly/Lac medium is not accompanied by detectable levels of $GAL4_{III}$. These and the previous results suggest that $GAL4_{III}$ might not be required for low to moderate *GAL/MEL* gene expression. Although it is possible that nondetectable levels of $GAL4_{III}$ contribute to the *GAL1* and *MEL1* expression in these cases, our results with *gal4* missense mutants (discussed below) militate against $GAL4_{III}$ being the only activating species of GAL4.

Three DNA-binding-competent *gal4* missense mutants lacking detectable GAL4 activity also lack detectable levels of $GAL4_{II}$. A fourth mutant, which retains only 5% of GAL4 activity, exhibits reduced levels of $GAL4_{II}$. Alternate substitutions at the same positions which restore activation competence to these completely inactive GAL4 proteins also restore phosphorylation (restore $GAL4_{II}$ or restore $GAL4_{II}$ and $GAL4_{III}$). Of particular note, higher levels of *GAL1* activation are realized in cells expressing *GAL4* revertant alleles for which $GAL4_{III}$-like mobility forms are detected.

Three of the four missense mutations that alter residues outside the DNA-binding domain (12, 24) and shown here to block phosphorylation of the GAL4 protein cluster within a 40-amino-acid region located roughly in the middle of the protein (20, 21). It is remarkable that none of the four falls within either activation region I or II (29, 31). Nevertheless, the fact that all four of these missense mutations affect transcription activation and affect the phosphorylation state of GAL4 supports the notion that this modification may be important for its function.

On the basis of our results, we suggest two testable hypotheses: (i) $GAL4_I$ is not capable of transcription activation but must undergo phosphorylation(s) to give rise to active species, and (ii) phosphorylation to $GAL4_{III}$ creates a more potent activator than phosphorylation to $GAL4_{II}$. To test for a direct causal relationship between phosphorylation and GAL4 protein activity, it will ultimately be necessary to directly compare the potencies of the different forms of

GAL4 as transcriptional activators in vitro. Currently, we cannot exclude the possibility that mobility-altering phosphorylations ($GAL4_I$ to $GAL4_{II}$ or $GAL4_{III}$) might only indicate unique conformations of GAL4 protein that are associated with various levels of GAL4-dependent transcription activation. Accordingly, such conformations of GAL4 (and the phosphorylations) would be prevented by the amino acids substitutions present in the four missense mutations.

Three of the four in vivo-selected *gal4* mutations alter serine residues (at positions 322, 352, and 511). Are phosphorylations at these serine residues required for $GAL4_{II}$ or $GAL4_{III}$ formation? $GAL4_{II}$ formation appears not to require phosphorylation at either Ser-322 or Ser-511. Substitutions Ser-322 → Phe and Ser-511 → Arg/Leu do not block $GAL4_{II}$ formation. We imagine that the inactivating substitutions at these positions prevent a conformation of the protein that is required for $GAL4_{II}$-like phosphorylation(s) at other sites. On the other hand, Ser-352 may be phosphorylated in $GAL4_{II}$, or it may be required for accurate $GAL4_{II}$-like phosphorylation, since we do not detect a well-defined $GAL4_{II}$-comigrating species for the (active) Ser-352 → Val allele. In the case of $GAL4_{III}$, formation does not require phosphorylation at Ser-511. Our data show that Ser-511 → Arg/Leu mutants do form $GAL4_{III}$. $GAL4_{III}$ formation may, however, require phosphorylation at position Ser-322, Ser-352, or both. Neither the Ser-322 → Phe mutant nor the Ser-352 → Val revertant exhibits detectable levels of $GAL4_{III}$, although both exhibit $GAL4_{II}$ (322) or $GAL4_{II}$-like (352) forms.

The diffuse band detected in the Ser-352 → Val revertant which migrates between the positions of $GAL4_I$ and $GAL4_{III}$ may reflect that multiple phosphorylations occur in the conversion of $GAL4_I$ to $GAL4_{II}$. If such is the case, the Ser-352 → Val electrophoretic variant(s) observed might represent incompletely phosphorylated molecules. This points out the potential complexity of GAL4 phosphorylation. Although we refer to $GAL4_{II}$ and $GAL4_{III}$ as if they are unique species, these bands may each represent closely migrating populations of phosphoisomers that are not resolved by our gels. The GAL4 protein sequence does contain several potential phosphorylation sites (33), and GAL4 may, like many other phosphorylation-controlled proteins, be subject to multiple phosphorylations by distinct protein kinases (39).

Our results with mutant BM792 (Lys-17 → Glu) indicate that neither UASgal binding nor *GAL/MEL* gene transcription is required for phosphorylation to $GAL4_{II}$. $GAL4_{III}$, however, is not detected in this mutant or in the other nonactive and less active, DNA-binding-competent missense mutants. Since the BM792-encoded protein could conceivably be defective in nuclear localization (35) as well as UASgal binding, the direct basis for the lack of $GAL4_{III}$ formation in this mutant is not known. In light of our results, we have noted from the work of others that $GAL4_{II}$ formation is apparently not required for nuclear accumulation. For example, a missense mutant of *GAL4* (Arg-51 → Gly) that was scored as competent in nuclear accumulation appeared as a single electrophoretic species after SDS-PAGE (44). In addition, none of several *GAL4-lacZ* hybrid proteins assayed showed evidence of electrophoretic heterogeneity, yet many appeared capable of nuclear accumulation (44).

Failure to bind to or activate transcription from the *GAL1*, *GAL10*, or *GAL7* promoters cannot be the sole reason for lack of $GAL4_{III}$ occurrence, since $GAL4_{III}$ is readily detected after galactose addition to cells lacking these genes (33). Moreover, $GAL4_{III}$ is detected in cells deleted of both *GAL3* and *GAL80* (Mylin and Hopper, unpublished data). Both *GAL3* and *GAL80* are transcriptionally activated by *GAL4* (2, 17, 43). Thus, $GAL4_{III}$ occurrence is not a direct consequence of transcription of *GAL1*, *GAL7*, *GAL10*, *GAL3*, or *GAL80*. Nevertheless, we cannot exclude the possibility that the transcriptional activation per se of other GAL4 protein-activated genes, such as *GAL2* (galactose permease [19]) and *GAL5* (phosphoglucomutase [36]) may cause or be required for the formation of $GAL4_{III}$.

In summary, GAL4 protein-dependent activation of *GAL/MEL* gene transcription occurs in cells that do not exhibit detectable levels of $GAL4_{III}$ but do contain $GAL4_{II}$. The levels of *GAL/MEL* gene expression measured in cells lacking $GAL4_{III}$, however, are lower than those found in cells that contain detectable levels of $GAL4_{III}$ under the same growth conditions. GAL4-dependent transcription activity is not detected in cells bearing *gal4* alleles for which GAL4 protein species corresponding to $GAL4_{II}$ or $GAL4_{III}$ are not observed. $GAL4_{II}$, like $GAL4_{III}$, is a phosphoprotein that can be converted by phosphatase treatment in vitro to $GAL4_I$. These results are consistent with the hypothesis (33) that differential phosphorylation of GAL4 protein produces physiologically relevant forms with differing activities. Recent advances toward developing a homologous in vitro transcription system that responds to added GAL4 derivatives may provide the means to directly test the functional significance of GAL4 phosphorylation.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant GM27925 (to J.E.H.) from the National Institutes of Health.

LITERATURE CITED

1. **Ammer, G.** 1983. Expression of genes in yeast using the *ADC1* promoter. Methods Enzymol. **101**:192–201.
2. **Bajwa, W., T. E. Torchia, and J. E. Hopper.** 1988. Yeast regulatory gene *GAL3*: carbon regulation; UASgal elements in common with *GAL1*, *GAL2*, *GAL7*, *GAL10*, *GAL80*, and *MEL1*; encoded protein strikingly similar to yeast and *Escherichia coli* galactokinases. Mol. Cell. Biol. **8**:3439–3447.
3. **Baker, S. M., S. A. Johnston, J. E. Hopper, and J. A. Jaehning.** 1987. Transcription of multiple copies of the yeast *GAL7* gene is limited by specific factors in addition to *GAL4*. Mol. Gen. Genet. **208**:127–134.

3a. **Bhat, P. J., D. Oh, and J. E. Hopper.** 1990. Analysis of the *GAL3* signal transduction pathway activating GAL4 protein-dependent transcription in *Saccharomyces cerevisiae*. Genetics **125**:281–291.

4. **Blume, K., and E. Beutler.** 1975. Galactokinase from human erythrocytes. Methods Enzymol. **42**:47–53.
5. **Bolivar, F., R. L. Rodriquez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. Gene **2**:95–113.
6. **Bostian, K. A., J. M. Lemire, L. E. Cannon, and H. O. Halverson.** 1980. *In vitro* synthesis of repressible yeast acid phosphatase: identification of multiple mRNAs and products. Proc. Natl. Acad. Sci. USA **77**:4504–4508.
7. **Botstein, D., S. C. Falco, S. E. Stuart, M. Brennan, S. Scherer, D. T. Stinchomb, K. Struhl, and R. W. Davis.** 1979. Sterile host yeasts (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene **8**:17–24.
8. **Boyer, K., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
9. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
10. **Bram, R. J., and R. D. Kornberg.** 1985. Specific binding to far

upstream activating sequences in polymerase II promoters. Proc. Natl. Acad. Sci. USA **82**:43–47.
11. **Bram, R. J., N. F. Lue, and R. D. Kornberg.** 1986. A family of upstream activating sequences in yeast: roles in both induction and repression of transcription. EMBO J. **5**:603–608.
12. **Brent, R., and M. Ptashne.** 1985. A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. Cell **43**:729–736.
13. **Carlson, M.** 1987. Regulation of sugar utilization in *Saccharomyces* species. J. Bacteriol. **169**:4873–4877.
14. **Giniger, E., S. M. Varnum, and M. Ptashne.** 1985. Specific DNA binding of GAL4, a positive regulatory protein of yeast. Cell **40**:767–774.
15. **Hess, H. H., M. B. Lees, and J. E. Derr.** 1978. A linear Lowry-folin assay for both water soluble and sodium dodecyl sulfate-solubilized proteins. Anal. Biochem. **85**:295–300.
16. **Hopper, J. E., J. R. Broach, and L. B. Rowe.** 1978. Regulation of the galactose pathway in *Saccharomyces cerevisiae*: induction of uridyl transferase mRNA and dependency on *GAL4* gene function. Proc. Natl. Acad. Sci. USA **75**:2878–2882.
17. **Igarashi, M., T. Segawa, Y. Nogi, Y. Suzuki, and T. Fukasawa.** 1987. Autogenous regulation of the *Saccharomyces cerevisiae* regulatory gene *GAL80*. Mol. Gen. Genet. **207**:273–279.
18. **Ito, H., Y. Fukudua, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
19. **Johnston, M.** 1987. A model fungal gene regulatory mechanism: the GAL genes of *Saccharomyces cerevisiae*. Microbiol. Rev. **51**:458–476.
20. **Johnston, M., and J. Dover.** 1987. Mutations that inactivate a yeast transcriptional regulatory protein cluster in an evolutionarily conserved DNA binding domain. Proc. Natl. Acad. Sci. USA **84**:2401–2405.
21. **Johnston, M., and J. Dover.** 1988. Mutational analysis of the *GAL4*-encoded transcriptional activator protein of *Saccharomyces cerevisiae*. Genetics **120**:63–74.
22. **Johnston, S. A., and J. E. Hopper.** 1982. Isolation of the yeast regulatory gene *GAL4* and analysis of its dosage effects on the galactose/melibiose regulon. Proc. Natl. Acad. Sci. USA **79**: 6971–6975.
23. **Johnston, S. A., M. I. Zavortnik, C. Debouck, and J. E. Hopper.** 1986. Functional domains of the yeast regulatory protein GAL4. Proc. Natl. Acad. Sci. USA **83**:6553–6557.
24. **Keegan, L., G. Gill, and M. Ptashne.** 1986. Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. Science **231**:699–703.
25. **Kuo, C.-L., and J. Campbell.** 1983. Cloning of *Saccharomyces cerevisiae* DNA replication genes: isolation of the *CDC8* gene and two genes that compensate for the *cdc8-1* mutation. Mol. Cell. Biol. **3**:1730–1737.
26. **Laughon, A., and R. F. Gesteland.** 1984. Primary structure of the *Saccharomyces cerevisiae GAL4* gene. Mol. Cell. Biol. **4**: 260–267.
27. **Lohr, D., and J. Hopper.** 1985. The relationship of regulatory proteins and DNase I hypersensitive sites in the yeast *GAL1-10* genes. Nucleic Acids Res. **13**:8409–8423.
28. **Lue, N. F., D. I. Chasman, A. R. Buchman, and R. D. Kornberg.** 1987. Interaction of *GAL4* and *GAL80* gene regulatory proteins in vitro. Mol. Cell. Biol. **7**:3446–3451.
29. **Ma, J., and M. Ptashne.** 1987. Deletion analysis of GAL4 defines two transcriptional activating segments. Cell **48**: 847–853.
30. **Ma, J., and M. Ptashne.** 1987. The carboxyl 30 amino acids of GAL4 are recognized by GAL80. Cell **50**:113–119.
31. **Ma, J., and M. Ptashne.** 1987. A new class of transcriptional activators. Cell **55**:443–446.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Mylin, L. M., J. P. Bhat, and J. E. Hopper.** 1989. Regulated phosphorylation and dephosphorylation of GAL4, a transcriptional activator. Genes Dev. **3**:1157–1165.
34. **Mylin, L. M., K. H. Hoffmann, L. D. Schultz, and J. E. Hopper.** 1990. A regulated GAL4 expression cassette providing controllable and high level output from high copy galactose promoters in yeast. Methods Enzymol. **185**:297–308.
35. **Nelson, M., and P. Silver.** 1989. Context affects nuclear localization in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9**: 384–389.
36. **Oh, D., and J. E. Hopper.** 1990. Transcription of a yeast phosphoglucomutase isozyme gene is galactose inducible and glucose repressible. Mol. Cell. Biol. **10**:1415–1422.
37. **Parthun, M. R., and J. A. Jaehning.** 1990. Purification and characterization of the yeast transcriptional activator *GAL4*. J. Biol. Chem. **265**:209–213.
38. **Post-Beittenmiller, M. A., R. W. Hamilton, and J. E. Hopper.** 1984. Regulation of basal and induced levels of the *MEL1* transcript in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4**: 1238–1245.
39. **Roach, P. J.** 1981. Glycogen synthase and glycogen synthase kinases. Curr. Top. Cell. Regul. **20**:45–105.
40. **Rothstein, R.** 1983. One step gene disruption in yeast. Methods Enzymol. **101**:202–211.
41. **Schultz, L. D., K. J. Hoffmann, L. M. Mylin, D. L. Montgomery, R. W. Ellis, and J. E. Hopper.** 1987. Regulated overproduction of the *GAL4* gene product greatly increases expression from galactose-inducible promotors on multicopy expression vectors in yeast. Gene **61**:123–133.
42. **Selleck, S. B., and J. E. Majors.** 1987. In vivo DNA-binding properties of a yeast transcriptional activator protein. Mol. Cell. Biol. **7**:3260–3267.
43. **Shimada, H., and T. Fukasawa.** 1985. Controlled transcription of the yeast regulatory gene *GAL80*. Gene **39**:1–9.
44. **Silver, P., A. Chiang, and I. Sadler.** 1988. Mutations that alter both localization and production of a yeast nuclear protein. Genes Dev. **2**:707–717.
45. **Torchia, T. E., R. W. Hamilton, C. L. Cano, and J. E. Hopper.** 1984. Disruption of the regulatory gene *GAL80* in *Saccharomyces cerevisiae*: effects on carbon-controlled regulation of the galactose/melibiose pathway genes. Mol. Cell. Biol. **4**: 1521–1527.
46. **Torchia, T. E., and J. E. Hopper.** 1986. Genetic and molecular analysis of the *GAL3* gene in the expression of the galactose/melibiose regulon of *Saccharomyces cerevisiae*. Genetics **113**: 229–246.
47. **Tschumper, G., and J. Carbon.** 1980. Sequence of a yeast DNA fragment containing a chromosomal replicator and the *TRP1* gene. Gene **10**:157–166.
48. **West, R. W., R. R. Yocum, and M. Ptashne.** 1984. *Saccharomyces cerevisiae GAL1-GAL10* divergent region: location and function of the upstream activating sequence UASg. Mol. Cell. Biol. **4**:2467–2478.
49. **Yocum, R. R., and M. Johnston.** 1984. Molecular cloning of the *GAL80* gene from *Saccharomyces cerevisiae* and characterization of a *gal80* deletion. Gene **32**:75–82.